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(54) Title: MONOCLONAL ANTIBODY TO ENTEROVIRUSES

(57) Abstract

A monoclonal antibody (designated 5-D8/1) is described which is produced by a hybridoma cell line and is capable of binding to an epitope of a VP1 protein of an enterovirus, which epitope is conserved throughout the group of viruses known as enteroviruses and excluding hepatitis A virus.

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MONOCLONAL ANTIBODY TO ENTEROVIRUSES

This invention relates to a monoclonal antibody to enteroviruses, a method for its production and its uses in assays for antigens of enteroviruses and for antibodies to enteroviruses in sera and other liquid samples and for the production and use of immunoabsorbents.

Enteroviruses are a group of viruses which proliferate in the gastrointestinal tract. These viruses are reported to contain single-stranded polycistronic RNA with positive polarity and to be approximately 27 nm in diameter. They exhibit cubic symmetry and have a buoyant density in CsCl of 1.34 g/ml, according to Andrews et al, "Viruses of vertebrates", 4th Edition, pp 1-37, Bailleric-Tindall, London (1978). Structurally the enteroviruses have four major virion polypeptides, VP1, VP2, VP3 and VP4. Enteroviruses are implicated in a variety of clinical conditions such as poliomyelitis, Bornholm disease, acute meningitis, acute myocarditis, diabetes mellitus type 1, orchitis and infections of the upper respiratory tract.

The group includes the polioviruses, Coxsackie A, Coxsackie B and the echoviruses. Some seventy-two distinct serotypes have been identified to date, with the recent addition of hepatitis A virus (HAV) to the group.

It has also been recently established that enterovirus infection is responsible for myalgic encephalomyelitis (ME), also known as "post-viral fatigue syndrome". Diagnosis of this infection has been hindered in the past because of the huge variety of enteroviruses for which no adequate diagnostic assay exists.

In the clinical situation, a separate characteristic assay is required for each of the various serotypes whereas, in practical terms, it is often the case that an assay for the group as a whole would suffice.

Enteroviruses are usually identified by neutralization employing intersecting antiserum pools using

combinations so designed that each serum is incorporated in more than one pool. Thus identification is usually slow, expensive, and tedious.

It is known that a protein sequence, known as VP1, is partially conserved throughout the whole of the enterovirus group. However, although it is possible to raise antibodies to that sequence, it has not been possible to produce an antibody which binds to all the serotypes: all the previous antibodies bound to VP1 epitopes and to epitopes on other protein systems which were not conserved throughout the whole group.

An object of the present invention is to provide a non-serotype-specific antibody for enteroviruses.

According to the present invention there is provided a monoclonal antibody 5-D8/1 binding to all serotypes of the enterovirus group, as herein defined.

The invention further provides a monoclonal antibody produced by a hybridoma cell line which is capable of binding to an epitope of a VP1 protein of an enterovirus, which epitope is conserved throughout the group of viruses known as enteroviruses and excluding hepatitis A virus. Preferably the hybridoma cell line is derived from a rodent, such as a mouse.

This invention is based, therefore, on the surprising discovery that, regardless of the indications derived from previous work, not only is the VP1 protein itself highly conserved throughout the group but that an epitope thereon is also conserved.

When tested against a wide range of enteroviral serotypes, the antibody of this invention has been found to bind, with the notable exception of the hepatitis A virus. However, this clear evidence of significant genetic variation from other members of the group, along with other recent indications from other unconnected research, tends to suggest that the HAV virus has been incorrectly placed in

the enterovirus group. Hence, in the context of the present specification, the definition "enterovirus" excludes the hepatitis A virus (HAV).

In a particularly preferred aspect of the present invention there is provided a monoclonal antibody designated 5-D8/1 that binds to all serotypes of the enterovirus group (as herein defined) and is secreted by the hybridoma cell line deposited on 14th April 1988 at European Collection of Animal Cell Cultures, PHLS Centre for Applied Microbiology & Research, Porton Down, Salisbury SP4 OJG, U.K., under Accession Number 88041401.

The present invention also provides a hybridoma cell line secreting the antibody of this invention, the said cell line having been deposited on 14th April 1988 at European Collection of Animal Cell Cultures under the Accession Number 88041401. The invention further extends to clones, sub-clones and cells having substantially the same properties as those of the hybridoma cell line deposited on 14th April 1988 at European Collection of Animal Cell Cultures under Accession Number 88041401.

We have furthermore found surprisingly that the monoclonal antibody designated 5-D8/1 is capable of binding to the VP1 protein of an enterovirus (as herein defined) despite the presence of human antibodies to the said VP1 protein. It thus appears that this monoclonal antibody has a paratope which is capable of binding to an epitope of a VP1 protein of an enterovirus, which epitope (i) is conserved throughout the group of enteroviruses (excluding hepatitis A virus) and (ii) is a different epitope from that to which a human antibody binds.

Also included within the scope of the invention is a hybrid antibody which comprises at least one fragment of a monoclonal antibody which has a paratope capable of binding to an epitope of a VP1 protein that is conserved throughout the group of enteroviruses, said at least one fragment being

selected from an Fab fragment, said paratope and an idiotype.

Normally it will be convenient to provide the monoclonal antibody with an immunometric marker. Hence the invention also embraces a labelled antibody comprising a monoclonal antibody which binds to an epitope of a VP1 protein of an enterovirus, which epitope is conserved throughout the VP1 proteins of all enteroviruses (excluding hepatitis A virus), which antibody has been labelled with an immunometric marker. Such an immunometric marker may be a fluorogenic marker, a luminometric marker, an enzymatic marker, a hapten marker, or a radioisotope. Introduction of a marker into the monoclonal antibody can be effected using standard techniques. Fluorogenic markers are described in US-A-3940475. Luminometric markers include luminol. Enzymatic markers are described in US-A-3645090. Horseradish peroxidase is a suitable enzymatic marker and may be conjugated with the antibody with the aid of cross-linking molecule, such as glutaraldehyde. Hapten markers include biotin. As examples of radioisotopes there can be mentioned the radioisotopes of iodine, such as ^{125}I.

Also provide in accordance with the present invention is an immunoabsorbent comprising a monoclonal antibody which is capable of binding to an epitope of a VP1 protein of an enterovirus, which epitope is conserved throughout the group of enteroviruses (as herein defined), and which has been bound to a solid phase. Standard techniques can be used to bind the antibody to the solid phase, which may be, for example, a filter paper, a polymer, such as nylon, polyethylene, polystyrene, or polypropylene, in bead or tube form or in the form of a well surface, for example a well surface of a multi-well plate. The solid phase may be a sugar, such as an agarose, or a modified sugar, such as a modified agarose, or a cross-linked dextran. Methods of binding antibodies to polysaccharide

supports are described in US-A-3645852.

We further provide a process for separating a VP1 protein of an enterovirus from a liquid medium containing same which includes the steps of:

(a) providing an immunoabsorbent comprising a monoclonal antibody which is capable of binding to an epitope of a VP1 protein of an enterovirus, which epitope is conserved throughout the group of enteroviruses, said monoclonal antibody having been bound to a solid phase;

(b) contacting the medium with the immunoabsorbent; and

(c) subsequently eluting the absorbed VP1 protein from the immunoabsorbent.

The invention further provides assays for enteroviruses, antigens thereof, and antibodies thereto in liquid samples such as sera and other biological fluids, utilising the said monoclonal antibody 5-D8/1.

The invention also envisages an immunocytochemical method for determining the presence or absence of an antigen of an enterovirus in a human tissue sample which comprises:

(a) providing a liquid medium containing an antibody labelled with an immunometric marker, said antibody having a paratope which is capable of binding to an epitope of a VP1 protein of an enterovirus, which epitope is conserved in the VP1 proteins of all enteroviruses and is different from the epitope of said VP1 protein to which human antibodies bind;

(b) incubating the tissue sample in the liquid medium;

(c) washing the incubated tissue sample; and

(d) determining the presence or absence of bound labelled antibody in the tissue sample, the presence of bound labelled antibody indicating the presence in the tissue sample of an antigen of an enterovirus.

A method for detecting the presence or absence of an antigen of an enterovirus in a fluid sample comprises:

(a) providing a monoclonal antibody produced by a

hybridoma cell line which is capable of binding to an epitope of a VP1 protein of an enterovirus, which epitope is conserved throughout the group of viruses known as enteroviruses and excluding hepatitis A virus;

(b) culturing tissue cells in the presence of the fluid sample;

(c) subjecting resulting cultured cells to fixation ;

(d) contacting the fixed cells with the monoclonal antibody of step (a); and

(e) monitoring the fixed cells for the presence or absence of bound antibody, the presence of bound antibody indicating the presence of an antigen of an enterovirus in the fluid sample.

In another aspect the invention provides a method of detecting the presence in a liquid sample taken from a patient of antibodies to an enterovirus which comprises:

(a) providing a liquid medium containing a first antibody which is a monoclonal antibody to a VP1 protein of an enterovirus that has a paratope which is capable of binding to an epitope of the said VP1 protein, which epitope (i) is conserved throughout the group of enteroviruses and (ii) is a different epitope from that to which a human antibody binds;

(b) contacting the liquid sample with an immobilised second antibody which is selected from an anti-IgG and an anti-IgM;

(c) washing the thus contacted immobilised second antibody;

(d) contacting the washed immobilised second antibody with a VP1 protein of an enterovirus;

(e) subsequently contacting the VP1 protein-treated immobilised second antibody with the liquid medium of step (a); and

(f) monitoring the resulting antibody-treated immobilised second antibody for the presence or absence of

bound first antibody, the presence of bound first antibody indicating the presence in the liquid sample of antibodies to an enterovirus. The liquid sample may be, for example, blood, serum, cerebral spinal fluid or a pericardial infusion. In this method a predetermined amount of the VP1 protein can be used in step (d), while a quantity of the liquid medium of step (a) is used in step (e) that contains a predetermined amount of the first antibody, so that the amount of bound first antibody observed in step (f) provides a measure of the level of antibodies to an enterovirus in the liquid sample.

We further provide a method of detecting an antigen of an enterovirus in a liquid sample which comprises:

(a) providing a first antibody which is a monoclonal antibody which is capable of binding to an epitope of a VP1 protein of an enterovirus, which epitope is conserved throughout the group of viruses known as enteroviruses and excluding hepatitis A virus;

(b) equilibrating a mixture of the liquid sample and a predetermined amount of the first antibody;

(c) providing a first substrate comprising an immobilised VP1 protein of an enterovirus;

(d) contacting the equilibrated mixture of step (b) with the first substrate of step (c);

(e) washing the thus contacted first substrate;

(f) providing a second substrate that comprises an immobilised VP1 protein of an enterovirus and that is substantially identical to the first substrate of step (c); and

(f) comparing the amount of bound first antibody on the washed first substrate of step (e) with a control sample obtained by contacting the second substrate of step (f) with a like predetermined amount of the first antibody to that used in step (b) and subsequently washing the thus contacted

second substrate, a reduction in the amount of bound first antibody on the first substrate compared with the amount of bound substrate on the second substrate providing a measure of the enterovirus antigen level in the liquid sample.

An immunoassay kit may thus comprise in separate containers:

(1) a monoclonal antibody to a VP1 protein of an enterovirus that has a paratope which is capable of binding to an epitope of the said VP1 protein, which epitope (i) is conserved throughout the group of enteroviruses and (ii) is a different epitope from that to which a human antibody binds;

(2) an immobilised second antibody which is selected from an anti-IgG and an anti-IgM; and

(3) a VP1 protein of an enterovirus.

An alternative immunoassay kit comprises in separate containers:

(1) a monoclonal antibody which is capable of binding to an epitope of a VP1 protein of an enterovirus, which epitope is conserved through the group of viruses known as enteroviruses and excluding hepatitis A virus; and

(2) a substrate comprising an immobilised VP1 protein of an enterovirus.

The various aspects of the present invention will now be described in the following description, by way of illustration.

Viruses and Mice

Virus stocks were prepared in various continuous cell lines and stored at -70°C. The viruses used were: Coxsackie viruses B1 to B6; echoviruses 11, 22 and 24; polioviruses 1, 2 and 3; Coxsackie viruses A7, 9 and 16; rhinovirus 1A; hepatitis A virus (HAV); yellow fever (YF); measles and rotaviruses.

All enterovirus strains, except HAV, were assayed by the plaque method on Hela S-3 cell monolayers grown on

plastic Petri dishes. Virus concentrations were defined as the number of PFU per millilitre of stock virus preparations. Infectivity assays of HAV were carried out using a quantitative enzyme-linked immunosorbent assay. The prototype strain of Coxsackie B5 [CB5 (Faulkner strain)] was used for the immunization of mice. The virus was purified by banding in two CsCl gradients. Female Balb/C mice were used.

Hybridoma Preparation

Aliquots of the virus containing 10^7 PFU/ml were heated at 56°C for 30 minutes and inoculated subcutaneously in complete Freund's Adjuvant (CFA) into female Balb/C mice. This dose was repeated two weeks later with incomplete adjuvant, and an aqueous booster dose was given intravenously three days prior to fusion.

Hybridoma cell lines were prepared by fusing parental mouse myeloma cells (NSO), with spleen lymphocytes from immunized mice by the technique of Kohler and Milstein [Nature, 256, 495-497 (1975)]. The hybridoma lines secreting antibody reactive with CB5 were identified by indirect immunofluorescence (IF) and cloned by the limiting dilution method of McKearn ["Monoclonal Antibodies", Plenum Press, New York, (1980), pp 403-404]. These clones were subsequently tested by IF against CB1, CB2, CB3, CB4, CB6, Coxsackie viruses A7(CA7), CA9, CA16, echoviruses 11, 22 and 24, polioviruses 1, 2 and 3, HAV, rhinovirus 1A, human rotavirus, measles virus, YF and adenovirus.

Indirect Immunofluorescence

The antigen substrate for IF was prepared by spotting trypsin-dispersed cells from infected cultures on to multispot glass slides. Uninfected cells were similarly treated as controls. These preparations were fixed in acetone for 20 minutes, dried and kept frozen until used. The spots were exposed to undiluted and 1:10 diluted hybridoma supernatants. Fluorescent anti-mouse

immunoglobulin was then used to detect bound mouse immunoglobulins (Ig) by an IF procedure of Gardner and McQuinn ["Rapid virus diagnosis, application of immunofluorescence", Butterworth, London (1974)].

Immunoblotting Assay

Cross-reactivity of the CB5 reactive monoclonal antibodies was confirmed by employing an enzyme immunobinding technique using the same range of viruses as those tested by IF with a modification of the method of Naz et al [Science, 225, 342-344 (1984)]. Briefly, a BioRad™ dot-blot apparatus was used to adsorb the antigens on to strips of nitrocellulose (NC) membrane. Residual binding sites were then saturated by blocking with 3% bovine serum albumin (BSA) in 0.05 M tris, 0.15 M NaCl, 0.5% Tween 20™ (pH 10.3). The strips were then incubated with the monoclonal antibody (5-D8/1), and after appropriate washing, they were incubated with peroxidase-conjugated rabbit anti-mouse gamma-globulins. The immunoblots were then developed with diaminobenzidine and hydrogen peroxide. Uninfected tissue culture cells and viruses other than enteroviruses were used as controls.

Western Blots

Western blots were used to delineate the epitopes recognised by the 5-D8/1 monoclonal antibody. Purified viral proteins were separated by SDS gel electrophoresis on 12.5% polyacrylamide gels [see Laemmli, Nature 227, pp 680-685 (1970)], transferred to NC membrane by electroelution, then processed for immunoblotting as described above. Biotinylated molecular weight markers were also electrophoresed, blotted and stained with peroxidase-conjugated streptavidin to verify the location of the reactive virion proteins.

Determination of the Monoclonal Antibody Isotypes

The subclass of the monoclonal antibodies in the

supernatant preparations was determined by double immunodiffusion using specific rabbit-anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgM.

Neutralization, Complement Fixation and Haemagglutination Inhibition Tests

A modification of the micro-titer neutralization test of Grist et al. ["Neutralisation Tests: in Diagnostic methods in clinical virology", pp 81-94, Blackwell, Oxford (1979)] was used. Briefly, serial twofold dilutions of 5-D8/1 immune ascitic fluid (50µl) were reacted with 100 PFU of the challenging virus (CB1, CB3 or CB5). Vero cells (1.5×10^4) were then added and the plate was incubated for 7 days at 37°C in 5% CO_2 in air. Titers were read microscopically as the highest dilution showing total protection against virus-specific cytopathic effect (CPE).

Microtiter plates were used to carry out complement fixation tests (CFT) and haemagglutination inhibition tests (HAI) by the method of Hawkes [Lennette, Schmidt, "Diagnostic procedures for viral, rickettsial and chlamydial infections": 5th Edition, pp 3-49, American Public Health Association, Washington (1979)]: 5-D8/1 immune ascitic fluid was used as the test antibody and rabbit hyperimmune sera as positive controls. Standardized CB1, CB3 and CB5 viruses were used an antigens. Titers for CFT were expressed as the highest dilution of antibody showing complete lysis of the indicator cells (sensitized sheep red blood cells). HAI titers were read as the highest dilution of antibody which totally inhibits agglutination of Type O human red blood cells.

RESULTS

Derivation of Hybridomas

A total of 108 hybridomas were derived in the course of six fusions. Of these, 75 showed no relevant specificity, while nine of them reacted with control Vero cells in the IF test and hence were disregarded. Fourteen

hybrid supernatants reacted specifically with Coxsackie virus B5 in the initial screening test, and only six of them retained activity on cloning at limiting dilutions. Four of these monoclonal antibodies had similar immunochemical properties and a wide range of reactivity with other enteroviruses. On this basis, one of them, 5-D8/1, was selected for further study. A hybridoma cell line secreting the antibody 5-D8/1 was deposited on 14th April 1988 at European Collection of Animal Cell Cultures under Accession Number 88041401.

Immunochemical Properties

Immunoglobulin isotype, as determined by gel diffusion, showed that 5-D8/1 is of the IgG_{2a} subclass. Western blots, carried out using a wide range of enteroviruses as well as viruses other than picornaviruses showed that the monoclonal antibody reacted with a single peptide having a relative molecular mass of 34,000 to 37,000. By using silver staining data, and the known electrophoretic mobility of enterovirus peptides [see Katze and Crowell, J. gen. Virol., 50, pp 357-367 (1980)], it is clear that 5-D8/1 antibody reacts with the VP1 peptide. Western blots showed that all the enteroviruses were reactive with 5-D8/1 antibody, and that HAV was the only exception. Control viruses (YF and measles, which are not enteroviruses) were negative.

Coxsackie viruses B3, B5, poliovirus 1, echoviruses 11, 22, YF and measles viruses were all partially purified and used at titers of 10^8 PFU/ml. HAV was also partially purified in comparable titers as assayed by the described method of Garelick et al. [Proceedings of the International Conference on Viral Hepatitis, London (1987)]. Coxsackie viruses A7 and A9 were used as tissue culture supernatants with titers of 10^6 PFU/ml, and this accounted for the relatively weaker bands in the Western blots.

Immunofluorescence and Enzyme Immunoassay

By IF, the 5-D8/1 antibody reacted with all of the enteroviruses tested, but did not react with unrelated viruses or cell controls. The results are summarised in Table 1 below. In quantitative terms, the ascitic fluid used gave titers ranging from 10^3 to 10^4 for the different enteroviruses.

TABLE I

Antigen	EIA titer	IF titer
CB1	5.0	3.7
CB2	5.5	4.0
CB3	4.5	3.7
CB4	5.0	3.0
CB5	6.0	4.0
CB6	3.5	2.5
CA9	3.9	3.0
CA7	4.5	3.0
Polio 1	6.0	4.0
Polio 2	6.0	3.5
HAV	1.0	< 1.0
Echo 11	3.5	2.5
Echo 22	4.5	3.0
Rotavirus (human)	< 1.0	< 1.0
Yellow Fever	< 1.0	< 1.0
Measles	< 1.0	< 1.0
Rhinovirus A1	< 1.0	< 1.0
Adeno 18	< 1.0	< 1.0
Uninfected vero	< 1.0	< 1.0

Notes to Table I:

1. The cross-reactivity was assayed by enzyme immunoassay (EIA) and indirect immunofluorescence (IF).
2. Titer expressed in reciprocal $\log_{10}$.

Enzyme immunoassay gave very clear results with no non-specific reactions and strong staining at high antibody dilutions. Quantitatively, this assay was more sensitive than IF, giving titers in the region of 10-fold or higher. Both assays confirmed the group specificity of this monoclonal antibody to the genus of enteroviruses. Again, HAV did not react in either of the two systems.

Biological Properties

Neutralization. The 5-D8/1 antibody was found not to neutralize CB1, CB3, CB5 viruses even at the lowest dilution of its immune ascitic fluid tested (1:10).

Complement Fixation. In CFT, the monoclonal antibody was shown to fix complement efficiently even when used at very high dilutions. The results are summarised in Table II below.

TABLE II

Antigen	Neutralizing Titer	
	5-D8/1 antibody	Specific Rabbit anti-serum
CB1	1.0	10^3
CB3	1.0	10^3
CB5	1.0	10^3

Antigen	CFT Titer	
	5-D8/1 antibody	Specific Rabbit anti-serum
CB1	3.5	10^3
CB3	4.0	10^3
CB5	3.8	10^3

Antigen	HAI Titer	
	5-D8/1 antibody	Specific Rabbit anti-serum
CB1	4.2	10^3
CB3	3.5	10^3
CB5	4.0	10^3

Note to Table II:

Three biological functions of antibodies were assessed: the ability to neutralize, fix complement and inhibit haemagglutination titers expressed in reciprocal $\log_{10}$.

Haemagglutination. The 5-D8/1 antibody showed specific inhibition of haemagglutination by CB1, CB3 and CB5. Table II shows that HAI tests recorded high titers of the monoclonal antibody immune ascitic fluid. Rabbit immune antisera to CB1, CB3 and CB5 viruses were included in the three biological tests as positive controls and the results

confirmed the validity of the 5-D8/1 data.

Discussion

Although the vast majority of enterovirus infections are subclinical and often pass unnoticed, it is now becoming increasingly clear that the contribution of these viruses to acute and chronic human diseases is significant [see King et al, Lancet i, pp 1397-1399 (1983) and Bowles et al Lancet i, pp 1120-1122 (1986)]. Since diseases associated with enteroviruses are not specifically related to certain serotypes, it may be assumed that the neutralization-specific epitopes themselves are not significantly, if at all, involved in pathogenesis. This concept of a group-specific approach to disease causation leads one to question the role of the group-specific epitopes in the initiation and possibly in the maintenance of the disease process.

Given the present complexity of the laboratory investigation of the enteroviruses in both research and diagnosis, it is believed that the enterovirus group-reactive antibody 5-D8/1 of this invention is a very useful analytical reagent. Although recent data have shown that the VP1 peptide contains the major common antigenic determinants of the Group B Coxsackie viruses, this invention shows that this holds true for all the enteroviruses tested, with the exception of HAV. This invention demonstrates that the relevant epitope of the VP1 protein which is recognised by the antibody 5-D8/1, is highly conserved, and this is consistent with recent genomic studies using cDNA probes derived from CB3 made by Tracy et al [Abstracts of the International Symposium on Inflammatory Heart Disease, p 80, European Society of Cardiology, Wurzburg (1987)].

That the antibody 5-D8/1 is not a neutralizing one was to be expected, since it is known that neutralizing antibody inducing epitopes are serotype-specific. The

efficient complement-fixing ability of 5-D8/1 antibody can be reliably exploited to identify enterovirus isolates in laboratories where the assay is more convenient. The antibody clearly has utility in enterovirus research and diagnostic work.

The application of the monoclonal antibody 5-D8/1 in a diagnostic method will now be described.

Identification of Clinical Isolates

In these tests, 130 different field isolates of enteroviruses were screened in duplicate by dot-blot enzyme-immunoassay by the method of Naz et al cited above. Eight clinical isolates of human adenovirus, two isolates of rotavirus, yellow fever virus and measles virus vaccine strains, as well as uninfected tissue culture cells, were included in the test as controls. After adsorption of the antigens on to strips of NC membrane, they were incubated first with 5-D8/1 antibody, and then with peroxidase-labelled rabbit anti-mouse gamma-globulin. The colour was then developed using diaminobenzidine and hydrogen peroxide, the strips were dried, and the colour density was read with a densitometer.

Detection of Antigens in Circulating Immune Complexes

Sera were obtained from 87 patients with chronic post-viral fatigue syndrome. Another group of sera was obtained from 32 patients with other diseases associated with the formation of immune complexes, for example, subacute bacterial endocarditis, systemic lupus erythematosus, and rheumatoid arthritis. A third group of sera was obtained from 35 normal individuals matched for sex, age and geographical location. The second and third groups acted as controls. All of the sera in the first and second groups were found previously to have circulating IgM complexes, as measured by the polyethylene glycol precipitation technique of Burton-Kee et al [J. clin. Path. 33: pp 653-659 (1980)]. To study the nature of the

complexed antigens a modification of the technique of Dambuyant et al. [Clin. exp. Path. 37: pp 424-432 (1979)] and of Zewdie [Ph.D. Thesis University of London (1983)] was used. Briefly, 10µl of undiluted peroxidase-conjugated 5-D8/1 antibody was added to 100µl of the patient's serum, and incubated for six days at 4°C in the presence of 0.02 mM EDTA. If the labelled antibody reacted with antigenic specificities in the IgM circulating immune complexes within the serum, then some of the labelled 5-D8/1 antibody became bound in the complex. After equilibration for six days, immune complexes were precipitated with polyethylene glycol at a final concentration of 2% in 10 mM EDTA. The amount of labelled antibody precipitated was then measured by adding substrate (4-aminophenazone and hydrogen peroxide) and measuring the colour spectrophotometrically.

Immunohistochemical Staining

Immunofluorescence. Fixed frozen myocardial sections from a child who died of a proven Coxsackie B4 myocarditis were tested by the indirect immunofluorescence method of Gardner and McQuinn cited above. Acetone-fixed frozen sections were washed in phosphate-buffered saline and then incubated with a 1:100 dilution of the 5-D8/1 antibody ascitic fluid in a moist chamber for 1 hour at 37°C: this was followed by application of fluorescein isothiocyanate-conjugated rabbit anti-mouse globulin. After further incubation for one hour at 37°C, sections were counterstained with Evans blue and mounted in glycerol.

Immunoperoxidase Staining

Formalin-fixed paraffin-embedded skeletal muscle autopsy material from CD-1 mice infected with Coxsackie virus B1 (Tucson stain), and Balb/C mice infected with Coxsackie virus A9 were tested by immunoperoxidase staining. Following dewaxing and rehydation, the staining was carried out by the technique of Norbert et al. Peroxidase activity was developed with diaminobenzidine and hydrogen peroxide by

the method described by Graham et al [J. Histochem. Cytochem. 14: pp 291-302 (1966)]. Sections were then counterstained with haematoxylin and mounted in DPX™ mountant.

RESULTS

Identification of Clinical Isolates

The results of identification by dot-blot enzyme immunoassays of the field isolates are shown in Figure 1. Each sample was run in duplicate, the mean value for each was calculated, and the $\log_{10}$ value of the mean plotted. The cutoff level for samples which were considered positive was taken as the mean of the control samples plus two standard deviations. It is evident from these results that 122 of the 130 clinical isolates (95%) were conveniently identified by the 5-D8/1 antibody as being enteroviruses. All of the control samples were negative in this test.

Detection of Antigens in Circulating Immune Complexes

The cross-reactions between the sera of patients and controls with the peroxidase-labelled enterovirus group-reactive antibody 5-D8/1 are shown in Figure 2. Sera of patients with the post viral fatigue syndrome demonstrated a significant increase in binding with the labelled 5-D8/1 antibody, compared with the control groups (χ^2 -24.1; $p < 0.01$). This showed that the complexes in these patients contained antigens which were the same as, or cross-reactive with, the enterovirus epitope against which the 5-D8/1 monoclonal antibody is directed.

In Situ Antigen Detection

By indirect immunofluorescence, the 5-D8/1 antibody selectively stained antigens in the post mortem heart sections obtained from a confirmed case of fatal acute myocarditis associated with Coxsackie virus B4 infection. Specificity was confirmed by using monoclonal antibody 14b in place of 5-D8/1. Immunofluorescence was confined to the cytoplasm of the individual myofibres, which is consistent

with the cytoplasmic replication of the virus.

By indirect immunoperoxidase staining, 5-D8/1 gave prominent reactions with Coxsackie viruses B1 and A9 antigens in mouse tissues. Group-reactive antigens were still detectable after 5 weeks of infection in a section from a mouse which developed myositis after being infected with Coxsackie virus B1 (Tucson strain). The ability of the antibody 5-D8/1 to detect Coxsackie virus A9 antigens in a mouse which developed fatal myositis after virus inoculation was clearly demonstrated. Control sections stained with monoclonal antibody 14b in place of 5-D8/1 did not show any peroxidase activity.

Discussion

The simple dot-blot enzyme immunoassay of this invention enabled 95% of field isolates to be identified. The remaining 5% of unidentified isolates were subsequently found to have low titers ($< 10^4$ plaque-forming units/ml) and were identified by 5-D8/1 antibody by an indirect immunofluorescence assay. The results clearly demonstrate that the relevant VP1 epitope which is recognised by 5-D8/1 monoclonal antibody is highly conserved. The fact that assays conducted using the 5-D8/1 antibody do not identify the specific serotype is not believed to be of great clinical significance.

The prime benefit of methods employed in detection and measurement of circulating immune complexes has been in following disease activity in immune complex-associated diseases, and monitoring the effect of treatment on the prognosis of such diseases. These methods have not, however, provided any diagnostic help about the nature of the antigens responsible for the disease. The slow reaction technique employed in this invention utilises the simple principle of reversibility of antigen/antibody reactions at equilibrium. This technique enables the identification of specific viral antigens present in circulating complexes and

thereby the identification of the infectious cause. The association of the enteroviruses with post-viral fatigue syndrome has been described [Behan et al. J. Infect. 105: pp 211-222 (1985) and McCartney et al. J. med. Virol. 19: pp 205-212 (1986)]. The test based upon monoclonal antibody 5-D8/1 show that about 50% of patients with this syndrome have IgM complexes which react strongly with the peroxidase-labelled 5-D8/1 antibody, indicating that the antigen in the complexes has specificity for the enterovirus group-reactive antibody.

There is little if any difficulty in staining fixed or frozen sections using either immunofluorescence or immunoperoxidase techniques. The group-reactive antibody 5-D8/1 makes an immunocytochemical approach to enterovirus studies a practical reality.

Purification and labelling of 5-D8/1 antibody

A pool of 630 ml of ascitic fluid from mice bearing the hybridoma was collected 14 days after inoculation with the hybridoma intraperitoneally. Single radial immunodiffusion was carried out in 2mm gels containing 1.5% rabbit anti-mouse IgG antiserum in 1% agarose in 10mM EDTA in 0.15 sodium chloride. 10 µl of ascitic fluid was put in 4 mm wells, and purified mouse IgG standards in other wells. The plates were incubated overnight at 37°C, and the diameter of the rings measured the next day. The total amount of IgG in the pool was calculated to be 245 mg. An affinity column of 12 ml of protein A - Sepharose (Pharmacia) was prepared in a small 15 mm diameter column, using a 10 mM EDTA/0.15M sodium chloride buffer, pH 7.6. The ascitic fluid was dialysed against the same buffer, and applied to the column. After all the fluid had passed through the column, the column was washed in the buffer until the effluent did not contain detectable protein. The column was then eluted with 0.1M sodium citrate/citric acid buffer, pH 3.0, and the protein

containing fraction collected, using a Pharmacia FPLC system. The eluate was concentrated by ultrafiltration, dialysed against 0.1M phosphate buffer, and the purity shown by immunoelectrophoresis against anti mouse serum. The final solution was stored in aliquots at -70°C until use.

Radiolabelling.

10 µg of the affinity purified 5-D8/1 was labelled with ^{125}I by the technique of Hunter & Greenwood ["Handbook of Immunological Methods", pp 17.2-17.12, ed Weir DM, Blackwell Scientific Publications, London (1974)] using 150µC of sodium iodide ($Na^{125}I$). The reaction product was dialysed exhaustively against phosphate buffered saline, and stored at -70°C until use. The specific activity of the final preparation in a volume of 2 ml, was 9000cpm/µl, or 1.8×10^6 cpm per µg.

Labelling with horse radish peroxidase

A two-stage method was used. 5 mg of horse radish peroxidase (HRP) was treated with a fourfold molar excess of redistilled glutaraldehyde, in 1.0 ml of 0.5M phosphate buffer pH 7.5. The free glutaraldehyde was removed by dialysis against phosphate buffered saline (PBS). The glutaraldehyde-HRP was added slowly to 12 mg of 5-D8/1 monoclonal antibody in 2 ml of PBS. The mixture was stirred at room temperature overnight. The remaining free glutaraldehyde groups were reacted with an excess of ethanolamine, and the final product dialysed against PBS. SDS PAGE electrophoresis showed that better than 95% of the HRP was bound to mouse IgG. The final solution was stored at -70°C until use.

VP1 protein

VP1 protein was prepared by the method of Werner et al [DNA, Volume 7, No. 5, pp 307-316 (1988)].

Immunoaffinity column

In order to purify VP1 preparations, a column of Sepharose 4B (Pharmacia) linked to the 5-D8/1 monoclonal

antibody was prepared. A suspension of 25 ml of Sepharose 4B was cooled to 4°C after equilibration with 0.1M Na_2CO_3. Small fragments of solid cyanogen bromide were added, until no more would dissolve. During the addition the pH was monitored, and 1.0M Na_2CO_3 added to maintain the pH at or above pH 9.5. At the end of the reaction, the gel was washed, by decantation, with large amounts of water, and then resuspended in an approximately 20% v/v suspension in 0.1M phosphate buffer, pH 7.5. 17 mg of the 5-D8/1 monoclonal antibody in the same buffer was added, and the solution was stirred at 4°C for 6 hours. The gel was then washed with PBS and poured into a 15 mm diameter column.

4.5 ml of tissue culture supernatant of a 6 day culture of Vero cells inoculated with 10^5 pfu Coxsackie B4 virus were heated at 56°C for 30 minutes, and treated with an equal volume of 0.05% sodium deoxycholate. The solution was clarified by centrifugation at 14,000g for 30 minutes. The supernatant was applied to the 5-D8/1 monoclonal antibody modified agarose column which was then washed with PBS until the effluent was free of protein. The column was then eluted with 0.1M citrate buffer pH 3.0 and 0.5 ml fractions collected.

The protein containing fractions were pooled and concentrated by ultrafiltration. 50 µl of the eluted fraction was electrophoresed in SDS PAGE, using BioRad™ electrophoresis equipment. Duplicate gels were prepared. One was stained with Aurodye™ for the protein bands, and the other transferred by electroelution to nitrocellulose membrane which was then blotted with HRP-labelled 5-D8/1 monoclonal antibody. There was one major band of 37 kd, with two very faint contaminant bands. Only the major band stained with HRP-labelled 5-D8/1 monoclonal antibody when counterstained with diaminobenzidine/hydrogen peroxide, indicating a high degree of purity of the eluted protein. One of the contaminant bands, with a molecular weight of 150

kd may represent small amounts of 5-D8/1 monoclonal antibody which were adsorbed, but not covalently linked to the column.

Immunohistochemical Staining for VP1 Antigen Detection

The monoclonal antibody 5-D8/1 can be used for detection of VP1 antigen in tissue samples by an immunohistochemical staining technique.

Tissue specimens

Tissue specimens of human origin were obtained as autopsy or biopsy material from patients with idiopathic polymyositis, PVFS, and acute myocarditis. Other tissue specimens were obtained from murine experimental models of polymyositis and myocarditis. Most of the samples were formalin-fixed and paraffin-embedded, and the rest were frozen.

Tissue samples which were fixed in buffered formalin (10% formalin in PBS at pH 7.4) were embedded in paraffin, cut 5 μm thick with a microtome blade and floated on a water bath. A pre-cleaned slide was thinly smeared with poly-l-lysine, as an adhesive, and the histologic sections were immediately picked up from the water bath. The excess water was drained and the slides were placed at 60°C for one hour, then stored away until used.

Cryostat sections (8μm) from frozen samples were mounted on coverslips and fixed for 20 minutes in cold acetone (-20°C) and kept frozen until used.

Indirect immunofluorescence (IF)

Cryostat sections of frozen samples were examined by immunofluorescence for the presence of enterovirus group-specific antigens as follows:

Sections were rehydrated in PBS and incubated in a 1:50 dilution of 5-D8/1 monoclonal Ab ascitic fluid in a moist chamber at room temperature for one hour. After washing in PBS for 20 minutes, FITC-conjugated rabbit anti-mouse gammaglobulin (1:50) was applied and incubated in a

moist chamber for one hour at room temperature. Just prior to mounting of these tissues in glycerol, they were again washed in PBS (ten minutes), then in distilled water (one minute), and counterstained for five seconds in Evan's blue.

Indirect immunoperoxidase

The tissue sections were deparaffinized through three changes of xylol and rehydrated through graded ethanol (74% and 64%) and brought to distilled water. They were placed in a Coplin jar that contained 0.1% trypsin (Sigma) and 0.1% calcium chloride in distilled water with the pH of the solution adjusted to 7.8, and incubated for thirty minutes at 37°C. The sections were thoroughly washed in PBS and digested tissue were encircled with a wax pencil to confine reagents and sera. Thereafter, the following steps were performed:

a - Bleach acid haematin with 7.5% H_2O_2 in distilled water for five minutes. Wash off with tap water.

b - Inhibit endogenous peroxidase with 2.2% periodic acid in distilled water for five minutes. Wash off with tap water.

c - Block aldehyde groups with 0.02% potassium borohydride in distilled water for two minutes. Wash off with PBS (pH 7.2) containing 0.02% sodium azide.

d - Incubate in moist chamber at room temperature with first antibody (mouse ascitic fluid or serum) diluted in 1% ovalbumin in PBS for thirty minutes. Wash off with PBS.

e - Agitate in PBS bath for fifteen minutes.

f - Incubate with normal rabbit serum diluted 1:10, to block Fc receptors, for fifteen minutes. Wash off with PBS.

g - Incubate at room temperature with HRP-labelled rabbit anti-mouse gammaglobulin diluted 1:100 in PBS for thirty minutes. Wash off with PBS.

The second antibody (enzyme-conjugated) is varied according to the species in which the first antibody was derived.

h - Agitate in PBS bath for fifteen minutes.

i - Incubate in diaminobenzidine, 5mg freshly dissolved in 10 ml 0.03% H_2O_2 in PBS. Wash with tap water as soon as brown pigments appear.

j - Counterstain with haematoxylin, dehydrate, and mount in DPX mountant.

Results

The monoclonal antibody 5-D8/1, direct labelled with horse radish peroxidase, or used in indirect immunoenzyme and immunofluorescent techniques, was used to study a variety of tissues potentially infected with enteroviruses. 9/15 children with unexplained myocarditis and 7/10 cells from the cerebrospinal fluid of children with 'viral meningitis' were positive. One childhood unidentified viral encephalitis was shown to be due to an enterovirus by staining a brain biopsy with 5-D8/1 antibody. In a study of chronic enterovirus infection of mice, samples of muscles obtained 5, 7, 14, 21, 28 and 35 days after infection showed specific staining for VP1 protein in each of 5 animals examined after each time period.

Detection of viral antigen in tissue cultures

The monoclonal antibody 5-D8/1 can also be used for detection of viral antigen in tissue cultures.

Indirect Immunofluorescence (IF)

The technique described by Gardner and McQuinn cited above was used with some modification. The antigen substrate for IF was prepared by spotting trypsin-dispersed cells, from infected and non-infected cell cultures, onto multispot glass slides, fixed in acetone for twenty minutes, and then dried and kept frozen until used. In the case of cerebrospinal fluid (CSF) specimens, cytospin preparations were fixed in acetone. The spots were exposed to undiluted

or 1:10 diluted hybridoma supernatant for thirty minutes in a humid chamber at 37°C. After washing in PBS, the same spots were exposed to 1:40 diluted fluorescein isothiocyanate-conjugated (FITC) rabbit anti-mouse gammaglobulin diluted 1:40, for thirty minutes in a humid chamber at 37°C, washed in PBS, counterstained with Evan's blue and then mounted in glycerol. This technique was used to screen hybrids supernatants, detect viral antigens in field specimens, and study cross reactivity of monoclonal antibodies.

Enzyme Immunobinding Assay (EIA)

A dot-blot EIA was performed with a modification of the technique described by Naz et al cited above. Briefly, a BioRad™ dot-blot apparatus was used to adsorb the antigens on strips of nitrocellulose (NC) membranes. Residual binding sites were saturated by blocking with 3% bovine serum albumin in 0.05 M tris, 0.15 M NaCl, 0.05% Tween 20™ (pH 10.3). The strips were incubated with monoclonal antibody 5-D8/1, and after appropriate washing they were incubated with a 1:200 dilution of peroxidase-conjugated rabbit anti-mouse gammaglobulin. The immunoblots were developed using 0.05% diaminobenzidine and 0.03% hydrogen peroxide in cold PBS, and colour density was scanned with a densitometer (Shimadzu - CS-930) and quantitated by integration of areas under peaks. This technique was applied to identify field isolates of enteroviruses, and to study cross-reactivity of the enterovirus group-specific monoclonal antibody 5-D8/1.

Detection of viral antigen in tissue cultures

148 cultures of isolates of enteroviruses as wild strain isolates from the population, were cultured in Vero cells, and the cultures stained with 5-D8/1 antibody, by indirect immunofluorescence using a fluorescent anti-mouse IgG as the second antibody. In 145/148 the staining revealed strong fluorescence of infected cells in the

culture, and in 25 control viral cultures no positives were seen. The results were obtained by an observer who did not know which viruses were in which culture. The identification of the enteroviral cultures was carried out on all 148 samples within one day, a result that could not be obtained by previously available methods.

Detection of VP1 in human blood samples

1. The first method involves addition of labelled antibody to serum, precipitating immune complexes, and measuring the fraction of labelled antibody in the precipitate.

In a case when the patient may have made antibody to the protein addition of labelled antibody to such serum may require a long time for equilibration to occur if the human and labelled added antibody have a similar specificity for epitopes of the viral antigen. (As we have subsequently found that the primate antibody and the mouse monoclonal antibody react with different epitopes, the time for equilibrium is not a problem. In case that were not so, an incubation time of 100 hours was used in this first detection method).

100µl of serum were mixed with 20 µg of peroxidase labelled 5-D8/1 monoclonal antibody, diluted in EDTA buffer, pH 7.6 in saline. The EDTA has a dual role, as buffer, and to prevent microbial growth during the incubation at 4°C for 100 hours. At the end of this time 500µl of 2.6% polyethylene glycol in EDTA saline was added, and after 4 hours at 4°C the precipitate was removed by centrifugation, and washed twice in 2 ml of 2% polyethylene glycol. The washed precipitate was redissolved in 500µl of EDTA saline and the samples transferred to 2 ml conical AutoAnalyzer™ cups. The peroxidase precipitated was measured in an AutoAnalyzer II™ using the standard phenol/aminophenazone technique, and hydrogen peroxide as substrate. The optical density was recorded and printed out as the percentage of

the colour that would be produced if all the 5-D8/1 antibody had been precipitated. This was calculated from dilutions of the 5-D8/1 antibody which were run as standards in the assay. Samples from up to 10 normal healthy subjects were added in each assay run, and the mean and standard deviation for the values for the normal samples were calculated. Serum samples above the mean plus two standard deviations for the normal controls were considered positive, that is having detectable amounts of VP1 in the serum. It will be seen that the ability to detect enteroviral VP1 antigen is high, and the assay, although labour intensive, can be applied routinely to detect infection. Using one and a half technicians, it is possible to carry out assays at a rate of 13,000 assays per year.

2. The second method for measuring circulating VP1 antigen depends on the use of a surface bound immunoassay. Such a technique can only be successful if the human and mouse monoclonal antibodies in fact react with separate sites on VP1. This was carried out thus:

The antisera tested were from naturally immune human subjects, rabbits immunised with recombinant VP1 from Coxsackie B3, and monkeys hyperimmunised with 7 doses of Coxsackie B1 and B3 virus.

Polystyrene 2.5 ml tubes were coated with 1 ml of a 1:500 dilution of purified VP1 in a 0.1M carbonate/0.1M bicarbonate buffer pH 9.21 for 1 hour. The washed tubes were then blocked with a 1% solution of haemoglobin in phosphate buffered saline for 30 minutes at room temperature. 2µl of radiolabelled 5-D8/1 antibody, diluted in 1 ml PBS, were mixed with 10µl of serum and incubated for 30 minutes at 37°C. The radioactive 5-D8/1 antibody/serum mixtures were then added to the VP1-coated tubes and incubated at 37°C for 1 hour. After washing 3 times with PBS, the bound radioactivity was then measured in a gamma counter. The results are set out in Table III.

Table III

Inhibition of binding of radiolabelled 5-D8/1 antibody (*D8) to VP1 coated tubes with antisera of different species.

Rabbit Anti-VP1	Counts Bound (cpm)
2µl *D8	3856, 2893
(2µl *D8 + 2µl NRS)	1890, 1787
(2µl *D8 + 2µl Rabbit anti-VP1)	4, 2
(2µl *D8 + uncoated, but blocked tube)	1, 4

Primate Sera	Counts Bound
(*D8 + coated tube)	3100, 2800
(*D8 + monkey anti-B3)	5800, 6100
(*D8 + monkey anti-B1)	6300, 5200
(*D8 + Human antiserum 1)	2700, 2100
(*D8 + Human antiserum 2)	2500, 2900

It will be seen from Table III that although immune rabbit serum produced marked inhibition of binding of the labelled 5-D8/1 antibody to the VP1, neither hyperimmune monkey serum, from animals immunised with three separate doses of enterovirus, nor adult human serum containing high levels of anti-enteroviral antibodies produced inhibition. In the case of the hyperimmune monkey sera the amount of binding actually increased. This result is explicable by the amount of rheumatoid factors present in such hyperimmune sera.

In neither human nor monkey sera was there any inhibition of binding of 5-D8/1 antibody, making it very likely that the two antibody specificities were for different epitopes on VP1. When rabbit, or mouse serum was used, however, there was dose dependent inhibition of binding, demonstrating that in this case the two antibodies were binding to the same, or very closely adjacent sites

such that the antisera produce steric hindrance of the binding of the labelled 5-D8/1 antibody.

Having established that the human sera containing antibodies did not inhibit the binding of 5-D8/1 antibody to VP1 coated tubes, the effect of the addition of VP1 was studied. It will be seen that there is dose dependent inhibition of binding of the monoclonal antibody to the tubes, unaffected by the presence of human antibody. Thus this assay should be able to detect VP1 antigen, and not detect human antibody. This is only possible if the test antibody reacts with a site different from the epitope which is recognised by human antibodies, and 5-D8/1 is a suitable antibody for this purpose.

Attempts were then made to measure the amounts of VP1 present in human sera by inhibition of binding of 5-D8/1 antibody to the coated tubes. The results were standardised by the use of control, normal sera to which were added graded quantities of VP1. The inhibition obtained could then be expressed in terms of equivalent amounts of VP1 added to the control sera which could produce equivalent inhibition. The results are listed in Table IV:

Table IV

Serum	Counts Bound
S1 alone	6448, 6979
S1 + 5µl VP1	2595, 3100
S1 + 10µl VP1	1637, 1487
S2 alone	8129, 8149
S2 + 2µl VP1	4055, 4830
S2 + 5µl VP1	3008, 2109
S2 + 10µl VP1	1634, 1291
no serum, no VP1	9100, 8100

In the experiments summarised in Table IV VP1 in serum was detected by inhibition of the binding of radiolabelled 5-D8/1 antibody to VP1 coated tubes. In each

case the serum sample with known amounts of pure VP1 added was mixed with radiolabelled 5-D8/1 antibody and then added to the coated tube. Radioactivity bound after 30 minutes incubation was recorded. Serum S1 was positive in the first method for VP1, and contained about 1µl of VP1. The serum alone value is thus reduced below that seen with serum S2 which does not contain detectable amounts of VP1.

The conditions for optimisation of the coated tube assay were determined by measuring the amounts of labelled 5-D8/1 antibody bound when different doses were added to the tubes, and optimisation of the coating conditions. These results are shown in Tables V and VI:

Table V

Effect of altering the concentration of VP1 used to coat tubes.

Dilution of VP1 Solution	Counts Bound
Undiluted	7540, 7752
1/10	4851, 4533
1/100	140, 73
1/1000	40, 18

In the tests of Table V 18000 cpm of radiolabelled 5-D8/1 antibody was used in each test.

Table VI

Binding of radiolabelled 5-D8/1 antibody to optimally VP1 coated tube.

Amount of Antibody	Total Counts Added	Counts Bound
1µl	3900, 3685	574, 553
2µl	8500, 8500	1732, 1128
3µl	13555, 13546	3158, 2081
4µl	17850, 18700	2010, 3569

These experiments illustrate the ability to use the monoclonal antibody to measure VP1 in human sera,

uninfluenced by the presence of human antibodies to VP1, which are normally found in human sera (vide infra).

Detection of human and monkey anti-VP1 antibodies and immunoglobulin classes in serum, using sandwich ELISA/RIA techniques

The method used was a modification of the ELISA technique of El-Hagrassy et al [Lancet ii: pp 1159-1162 (1980)] replacing the polyclonal rabbit anti-enteroviral detector antibody with direct peroxidase-labelled 5-D8/1 antibody. The experiment then involves coating the microtiter plate with anti-human IgG or anti-human IgM antibody, adding the human sera to the plate, and binding the human immunoglobulin. Enteroviral antibodies in the human immunoglobulin are able to capture antigens from an enteroviral preparation, and this in turn binds the detector antibody to measure the amount of viral antigen bound.

This test, using anti-human IgM, was carried out in duplicate on sera, with the detector antibody being either the polyvalent rabbit serum raised against Coxsackie B viruses, or the monoclonal antibody 5-D8/1. The results are set out in Table VII below:

Table VII

Antigen	Adults and Children > 5 yrs		Children > 5 yrs	
	Rabbit Polyclonal Antibody	5-D8/1 Antibody	Rabbit Polyclonal Antibody	5-D8/1 Antibody
CB1	14/14	14/14	6/6	2/6
CB2	17/17	15/17	3/3	0/3
CB3	15/15	15/15	5/5	2/5
CB4	16/16	15/16	4/4	2/4
CB5	18/18	18/18	2/2	2/4
Sub Total	80/80	77/80	20/20	8/20

As will be seen from Table VII, in adult sera the 5-D8/1 antibody detected as many positives as the polyclonal antibody, but in young children there were examples when the standard assay identified children with monotypic responses to the serotype specific antigens. Some of the young children indicated to be positive by the standard serum are negative using 5-D8/1 monoclonal antibody as their antibodies are not directed against VP1 protein. In adults the heterotypic response against VP1 protein is present, so that 'false negatives' are found largely in the children under 5 years old. It appears therefore that by the time exposure has been obtained to several enteroviruses (by age 5-6 years), all individuals are making antibody to the VP1 protein. The standard assay would miss some antibodies in people making immune responses to viruses separate from the serotypes overlapping Coxsackie B. More total positives are found using 5-D8/1 antibody than the polyclonal rabbit antiserum.

In this assay 5-D8/1 antibody can detect enteroviral infection, or even immunisation with poliovirus, and will be expected to detect all antibodies missed by the standard methods. The use of a standard reproducible reagent, rather than repeated production of dissimilar rabbit polyclonal antisera, must make for better standardisation of testing and consistency of results.

International Application No: PCT/GB 89/00386

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 3, line 4 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

European Collection of Animal Cell Cultures

Address of depositary institution (including postal code and country) [4]

PHLS Centre for Applied Microbiology & Research
Porton Down, Salisbury SP4 OJG, U.K.

Date of deposit [5]	Accession Number [6]
14th April 1988 (14.04.88)	88041401

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC).

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)

David Brown

(Authorized Officer) ROOM 5/6

INTERNATIONAL UNIT EXT 6759

☐ The date of receipt (from the applicant) by the International Bureau [10] was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

(January 1985)

CLAIMS:

1. A monoclonal antibody which is capable of binding to all serotypes of the enterovirus group.

2. A monoclonal antibody produced by a hybridoma cell line which is capable of binding to an epitope of a VP1 protein of an enterovirus, which epitope is conserved throughout the group of viruses known as enteroviruses and excluding hepatitis A virus.

3. A monoclonal antibody according to claim 2, in which the hybridoma cell line is derived from tissue from a rodent.

4. A monoclonal antibody according to claim 3, in which the hybridoma cell line is derived from tissue from a mouse.

5. A monoclonal antibody designated 5-D8/1 that binds to all serotypes of the enterovirus group and is secreted by the hybridoma cell line deposited on 14th April 1988 at European Collection of Animal Cell Cultures under Accession No. 88041401.

6. A monoclonal antibody which is capable of binding to the VP1 protein of an enterovirus despite the presence of human antibodies to the said VP1 protein.

7. A monoclonal antibody which is capable of binding to any enterovirus selected from Coxsackie viruses B1 to B6, echoviruses 11, 22 and 24, polioviruses 1, 2 and 3, and Coxsackie viruses A7, A9 and A16.

8. A monoclonal antibody which is capable of binding

to the VP1 protein of any enterovirus selected from Coxsackie viruses B1 to B6, echoviruses 11, 22 and 24, polioviruses 1, 2, and 3, and Coxsackie viruses A7, A9 and A16.

9. A monoclonal antibody to a VP1 protein of an enterovirus that has a paratope which is capable of binding to an epitope of the said VP1 protein, which epitope (i) is conserved throughout the group of enteroviruses and (ii) is a different epitope from that to which a human antibody binds.

10. A hybrid antibody comprising at least one fragment of a monoclonal antibody which has a paratope capable of binding to an epitope of a VP1 protein that is conserved throughout the group of enteroviruses, said at least one fragment being selected from an Fab fragment, said paratope and an idiotype.

11. A labelled antibody comprising a monoclonal antibody which binds to an epitope of a VP1 protein of an enterovirus, which epitope is conserved throughout the VP1 proteins of all viruses of the group of enteroviruses, which antibody has been labelled with an immunometric marker.

12. A labelled antibody according to claim 11, in which the immunometric marker is selected from fluorogenic markers, luminometric markers, enzymatic markers, hapten markers, and radioactive isotopes.

13. A labelled antibody according to claim 11, in which the marker comprises horseradish peroxidase.

14. A labelled antibody according to claim 11, in which the marker comprises a radioactive isotope of iodine.

15. An immunoabsorbent comprising a monoclonal antibody which is capable of binding to an epitope of a VP1 protein of an enterovirus, which epitope is conserved throughout the group of enteroviruses, said antibody having been bound to a solid phase.

16. An immunoabsorbent according to claim 15, in which the solid phase is selected from filter papers, polymers, sugars, modified sugars, and cross-linked dextrans.

17. An immunoabsorbent according to claim 16, in which the solid phase is selected from agaroses and modified agaroses.

18. An immunoabsorbent according to claim 14 in the form of beads.

19. An immunoabsorbent according to claim 14 in the form of a tube.

20. An immunoabsorbent according to claim 14 in the form of a well surface.

21. The hybridoma cell line deposited on 14th April 1988 at European Collection of Animal Cell Cultures under Accession No. 88041401.

22. Clones, subclones and cells having substantially the same properties as those of the hybridoma cell line deposited on 14th April 1988 at European Collection of Animal Cell Cultures under Accession No. 88041401.

23. A process for separating a VP1 protein of an enterovirus from a liquid medium containing same which

includes the steps of:

(a) providing an immunoabsorbent comprising a monoclonal antibody which is capable of binding to an epitope of a VP1 protein of an enterovirus, which epitope is conserved throughout the group of enteroviruses, said monoclonal antibody having been bound to a solid phase;

(b) contacting the medium with the immunoabsorbent; and

(c) subsequently eluting the absorbed VP1 protein from the immunoabsorbent.

24. An immunocytochemical method for determining the presence or absence of an antigen of an enterovirus in a human tissue sample which comprises:

(a) providing a liquid medium containing an antibody labelled with an immunometric marker, said antibody having a paratope which is capable of binding to an epitope of a VP1 protein of an enterovirus, which epitope is conserved in the VP1 proteins of all enteroviruses and is different from the epitope of said VP1 protein to which human antibodies bind;

(b) incubating the tissue sample in the liquid medium;

(c) washing the incubated tissue sample; and

(d) determining the presence or absence of bound labelled antibody in the tissue sample, the presence of bound labelled antibody indicating the presence in the tissue sample of an antigen of an enterovirus.

25. A method according to claim 24, in which the immunometric marker is selected from fluorogenic markers, luminometric markers, enzymatic markers, hapten markers, and radioactive isotopes.

26. A method according to claim 24, in which the immunometric marker is horse radish peroxidase.

27. A method according to claim 24, in which the immunometric marker is a radioactive isotope of iodine.

28. A method for detecting the presence or absence of an antigen of an enterovirus in a fluid sample which comprises:

(a) providing a monoclonal antibody produced by a hybridoma cell line which is capable of binding to an epitope of a VP1 protein of an enterovirus, which epitope is conserved throughout the group of viruses known as enteroviruses and excluding hepatitis A virus;

(b) culturing tissue cells in the presence of the fluid sample;

(c) subjecting resulting cultured cells to fixation;

(d) contacting the fixed cells with the monoclonal antibody of step (a); and

(e) monitoring the fixed cells for the presence or absence of bound antibody, the presence of bound antibody indicating the presence of an antigen of an enterovirus in the fluid sample.

29. A method according to claim 28, in which the monoclonal antibody of step (a) is labelled with an immunometric marker.

30. A method according to claim 29, in which the immunometric marker is selected from fluorogenic markers, luminometric markers, enzymatic markers, hapten markers, and radioactive isotopes.

31. A method according to claim 30, in which the immunometric marker is horse radish peroxidase.

32. A method according to claim 30, in which the immunometric marker is a radioactive isotope of iodine.

33. A method of detecting the presence in a liquid sample taken from a patient of antibodies to an enterovirus which comprises:

(a) providing a liquid medium containing a first antibody which is a monoclonal antibody to a VP1 protein of an enterovirus that has a paratope which is capable of binding to an epitope of the said VP1 protein, which epitope (i) is conserved throughout the group of enteroviruses and (ii) is a different epitope from that to which a human antibody binds;

(b) contacting the liquid sample with an immobilised second antibody which is selected from an anti-IgG and an anti-IgM;

(c) washing the thus contacted immobilised second antibody;

(d) contacting the washed immobilised second antibody with a VP1 protein of an enterovirus;

(e) subsequently contacting the VP1 protein-treated immobilised second antibody with the liquid medium of step (a); and

(f) monitoring the resulting antibody-treated immobilised second antibody for the presence or absence of bound first antibody, the presence of bound first antibody indicating the presence in the liquid sample of antibodies to an enterovirus.

34. A method according to claim 33, in which the first antibody of step (a) is labelled with an immunometric marker.

35. A method according to claim 34, in which the immunometric marker is selected from fluorogenic markers, luminometric markers, enzymatic markers, hapten markers, and radioactive isotopes.

36. A method according to claim 34, in which the immunometric marker is horse radish peroxidase.

37. A method according to claim 34, in which the immunometric marker is a radioactive isotope of iodine.

38. A method according to claim 33, in which a predetermined amount of the VP1 protein is used in step (d), in which a quantity of the liquid medium of step (a) is used in step (e) that contains a predetermined amount of the first antibody, and in which the amount of bound first antibody observed in step (f) provides a measure of the level of antibodies to an enterovirus in the liquid sample.

39. A method of detecting an antigen of an enterovirus in a liquid sample which comprises:

(a) providing a first antibody which is a monoclonal antibody which is capable of binding to an epitope of a VP1 protein of an enterovirus, which epitope is conserved throughout the group of viruses known as enteroviruses and excluding hepatitis A virus;

(b) equilibrating a mixture of the liquid sample and a predetermined amount of the first antibody;

(c) providing a first substrate comprising an immobilised VP1 protein of an enterovirus;

(d) contacting the equilibrated mixture of step (b) with the first substrate of step (c);

(e) washing the thus contacted first substrate;

(f) providing a second substrate that comprises an immobilised VP1 protein of an enterovirus and that is substantially identical to the first substrate of step (c); and

(f) comparing the amount of bound first antibody on the washed first substrate of step (e) with a control sample obtained by contacting the second substrate of step (f) with

a like predetermined amount of the first antibody to that used in step (b) and subsequently washing the thus contacted second substrate, a reduction in the amount of bound first antibody on the first substrate compared with the amount of bound substrate on the second substrate providing a measure of the enterovirus antigen level in the liquid sample.

40. A method according to claim 39, in which the first antibody of step (a) is labelled with an immunometric marker.

41. A method according to claim 40, in which the immunometric marker is selected from fluorogenic markers, luminometric markers, enzymatic markers, hapten markers, and radioactive isotopes.

42. A method according to claim 40, in which the immunometric marker is horse radish peroxidase.

43. A method according to claim 40, in which the immunometric marker is a radioactive isotope of iodine.

44. An immunoassay kit comprising in separate containers:

(1) a monoclonal antibody to a VP1 protein of an enterovirus that has a paratope which is capable of binding to an epitope of the said VP1 protein, which epitope (i) is conserved throughout the group of enteroviruses and (ii) is a different epitope from that to which a human antibody binds;

(2) an immobilised second antibody which is selected from an anti-IgG and an anti-IgM; and

(3) a VP1 protein of an enterovirus.

45. An immunoassay kit comprising in separate

containers:

(1) a monoclonal antibody which is capable of binding to an epitope of a VP1 protein of an enterovirus, which epitope is conserved through the group of viruses known as enteroviruses and excluding hepatitis A virus; and

(2) a substrate comprising an immobilised VP1 protein of an enterovirus.

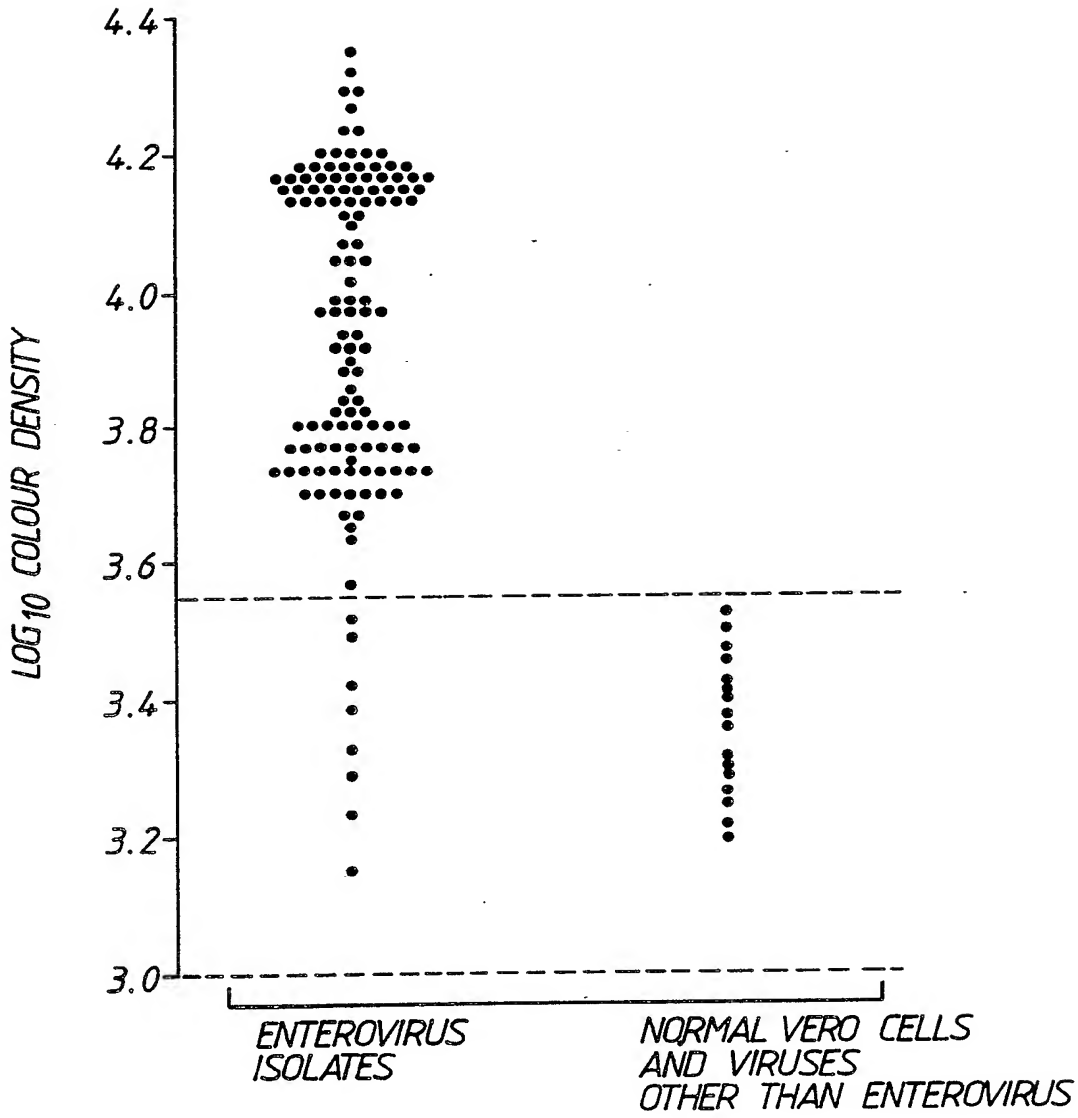
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
LOG10 COLOUR DENSITY
ENTEROVIRUS ISOLATES
NORMAL VERO CELLS AND VIRUSES OTHER THAN ENTEROVIRUS

Fig.1.

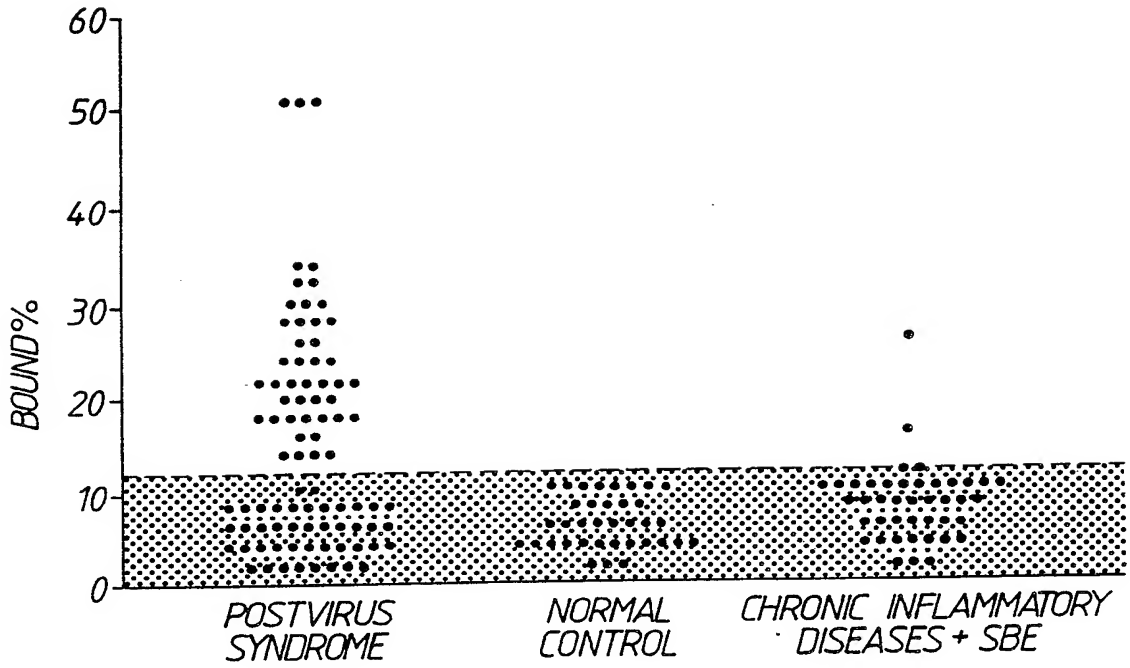
60
50
40
30
20
10
0
BOUND%
POSTVIRUS SYNDROME
NORMAL CONTROL
CHRONIC INFLAMMATORY DISEASES + SBE

Fig.2.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/00386

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 07 K 15/00, C 12 P 21/00, C 12 N 5/00, C 12 N 15/00, C 07 K 3/18, G 01 N 33/569, //(C 12 P 21/00, C 12 R 1:91)

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	A 61 K; C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Biological Abstracts, volume 86, no. 1, 1988, (Philadelphia, PA, US), G.E. Yousef et al.: "Derivation and biochemical characterization of an enterovirus group-specific monoclonal antibody", see page AB-1241, abstract 10907, & Intervirology 28(3): 163-170, 1987 --	1-23
X	Biological Abstracts, volume 86, no. 2, 1988, (Philadelphia, PA, US), G.E. Yousef et al.: "Clinical and research application of an enterovirus group-reactive monoclonal antibody", see page AB-1306, abstract 22384, & Intervirology 28(4): 199-205, 1987 (1988) --	1,2,21,22, 24-45
X	Biological Abstracts/Reports-Reviews-Meetings, volume 34, no. 103316, 1988, G.E. Yousef et al.: "Lack of reactivity of hepatitis A virus with two entero- /	1,2

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30th June 1989	26. 07. 89
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

International Application No. PCT/GB 89/00386

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	virus group-specific reagents a monoclonal antibody and a complementary DNA probe", see title and terms, & Journal of Medical Microbiology (England) 1987, vol 24, no 4, pII --	
A	WO, A, 83/03972 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 24 November 1983 see page 6, line 2 - page 7, line 30; page 10, line 19 - page 11, line 5; claims 1,12,23	1-45

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 8900386 SA 28058

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 17/07/89
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8303972	24-11-83	DE-A- 3374170	03-12-87
		EP-A,B 0107708	09-05-84